

# Viability and *bar* expression are negatively correlated in Oregon Wolfe Barley Dominant hybrids

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## Summary

The expression level of *bar*, which encodes phosphinothricin acetyltransferase (PAT), was correlated with the inviability of barley hybrids between 20 Golden Promise-derived transgenic lines (*Ds-bar* lines) and a specialized genetic marker stock, Oregon Wolfe Barley Dominant (OWBD). Each *Ds-bar* line was homozygous for a modified maize *Ds* element that encoded *bar* and that had been delivered via transposition to a unique location. All *Ds-bar* lines were viable and morphologically similar. Only four of the 20 hybrid populations were viable. The remaining populations died prior to producing seed. Phenotypic, enzyme-linked immunosorbent assay and quantitative reverse transcriptase-polymerase chain reaction analyses of these lines, and of lines from unrelated transformation events that also expressed *bar*, showed that viability was negatively correlated with *bar* expression. Analysis of crosses of a high-*bar*-expressing line with the OWB mapping population showed that the sensitivity of OWBD to PAT segregated as a single locus on chromosome 6HL. No sensitivity to PAT could be detected in several other lines and cultivars. OWBD has been shown to be genetically divergent from other germplasm groups within cultivated barley; therefore, the observed sensitivity may be peculiar to OWBD and thus would not impact generally on the utility of *bar* as a selectable marker or source of herbicide resistance in barley. Nevertheless, these results demonstrate the extent of allelic variability present in *Hordeum vulgare*, and suggest an additional variable for consideration when devising protocols for the transformation of *Hordeum* cultivars or landraces that are not known to be tolerant to PAT.

**Keywords:** *bar*, *Hordeum vulgare* L., phosphinothricin acetyltransferase, transgenic barley.

## Introduction

The development of routine transformation techniques for monocot species has been facilitated by several technical advances, including the development of biolistic transformation (Klein *et al.*, 1987, 1988; Christou, 1992) and the identification of suitable selectable markers, i.e. those that enable the efficient recovery of fertile plants from transgenic cells while suppressing the recovery of plants from non-transgenic cells. An additional critical feature of such markers is that, except for conferring tolerance to a selective agent, they do not appear to interact with the host metabolism. The *Streptomyces hygroscopicus*-derived selectable marker *bar* (Thompson *et al.*, 1987) has

been used widely for the production of transgenic barley, wheat, rice, maize, oat and other monocot species (Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990; Christou *et al.*, 1991; Somers *et al.*, 1992; Weeks *et al.*, 1993; Wan and Lemaux, 1994; Zhong *et al.*, 1996; Cheng *et al.*, 1997; Tingay *et al.*, 1997). The protein encoded by *bar*, phosphinothricin acetyltransferase (PAT), acetylates and thereby detoxifies phosphinothricin (glufosinate; 4-[hydroxy(methyl)phosphinoyl]-DL-homoalanine) and related compounds, such as the herbicide glufosinate-ammonium. In addition to its utility for the identification of primary transgenics, *bar*-derived herbicide resistance enables the rapid screening of transgenic populations for the identification of homozygous lines and for the

study of transgene inheritance patterns (Bregitzer and Tonks, 2003). *Bar*-derived herbicide resistance has agronomic application (LibertyLink® maize and canola; Bayer CropScience, <http://www.bayercropscienceus.com>).

In some cases, self-pollinated derivatives of transgenic cereals that express *bar* have shown reduced performance, but several studies have attributed such reductions to somaclonal variation and not to transgene insertion and/or expression (Bregitzer *et al.*, 1998, 2006; Barro *et al.*, 2002). However, in this study, we describe an instance in which it appears that *bar* expression at high levels is associated with severe physiological disturbances. Prior to describing the experiments on which this conclusion is based, further background information must be presented to establish the context which led to this research.

As part of the development of transposon tagging resources for barley, *Hordeum vulgare* ssp. *vulgare* (McElroy *et al.*, 1997; Koprek *et al.*, 2000; Cooper *et al.*, 2004; Singh *et al.*, 2006), 20 transgenic Golden Promise lines (*Ds-bar* lines), each containing a modified *Ds* element in a unique location, were crossed with Oregon Wolfe Barley Dominant (OWBD) multiple marker stock (Costa *et al.*, 2001). Each of the *Ds* elements consisted of ubiquitin-driven *bar* flanked by the terminal sequences essential for transposition (Coupland *et al.*, 1988; Varagona and Wessler, 1990). None of the *Ds-bar* lines exhibited morphological abnormalities, yet only four of 20 hybrid progeny populations were viable. Progeny from the other populations showed moderate to severe reductions in growth rate, chlorosis (which initially appeared to be slight but which progressively became severe) and a lack of necrotic lesions. Ultimately, leaf tissues became senescent, leading to plant death either at the seedling stage or at some point prior to flowering (P. Bregitzer, unpubl. data). Several hypotheses were considered as potential explanations for this phenomenon, but only one withstood scrutiny: that OWBD had a limited tolerance for *bar*-encoded PAT. This report details the experiments that were conducted to establish the relationship between inviability and *bar* expression in OWBD.

## Results

### Phenotypic analyses

To provide an initial test of the hypothesis that the differential viability of OWBD/*Ds-bar* hybrids was associated with differential *bar* expression, OWBD was crossed with several barley lines (GP-*bar* lines) known to differ for *bar* expression (measured by resistance to glufosinate-ammonium). These

crosses also functioned to investigate the possibility that some factor – other than *bar* expression – common to certain *Ds-bar* lines caused the inviability, as the GP-*bar* lines were derived from transformation events unrelated to those that produced the *Ds-bar* lines, and did not contain any *Ac transposase* or *Ds* sequences. Crosses of OWBD with GP-*bar* 31 (lowest *bar* expression) and GP-*bar* 30 (intermediate *bar* expression) produced hybrids that developed and matured normally. Crosses of OWBD with GP-*bar* 59 (highest *bar* expression) produced hybrids that survived to flowering, but died prior to the formation and maturation of seed.

Phenotypic observations were made of the eight *Ds-bar* lines selected for detailed study and of their non-transgenic parent, cv. Golden Promise. The appearance of all *Ds-bar* lines was similar, although they were slightly less vigorous than Golden Promise. This observation was unremarkable and consistent with previous observations of transgenic barley, where reduced vigour and productivity have been attributed to somaclonal variation (Bregitzer *et al.*, 1998). Thus, if the observed hybrid inviability resulted from the intolerance of *bar* expression, these data suggested that the effects might be caused by a physiological interaction that did not occur in Golden Promise. Evidence that this interaction might be rare was obtained from observations of hybrid progeny derived from crosses of various *Ds-bar* lines with Oregon Wolfe Barley Recessive (OWBR) genetic stock, cv. Baroness and cv. Garnet. All progeny from these crosses developed and matured normally.

### Immunological analyses

To discover whether differential hybrid viability was associated with the amount of PAT protein in the GP-*bar* and *Ds-bar* lines and in their hybrids, enzyme-linked immunosorbent assays (ELISAs) were conducted. Protein extracts of *Ds-bar* lines 11, 18 and 19, which produced inviable hybrids, had an average absorbance value of 2.98. This compared with a value of 0.22 for *Ds-bar* lines 1, 6, 24 and 35, which produced viable hybrids (absorbance values based on 0.5 mg/mL total plant protein). The relative amounts of PAT protein in *Ds-bar* lines were heritable: hybrids of Garnet with *Ds-bar* lines 11, 19 and 21 showed an average absorbance value of 2.46. This compared with a value of 0.48 for hybrids of OWBD and Garnet with *Ds-bar* lines 1, 6, 24 and 35.

Analyses of *Ds-bar* lines derived from the low-expressing *Ds-bar* line 24 provided further evidence of a link between inviability and high *bar* expression. All hybrids of OWBD and eight *Ds-bar* line 24 derivatives (in which *Ds* was transposed to a new location) were inviable. Protein extracts from these

**Table 1** Phosphinothricin acetyltransferase (PAT) protein concentrations (ng/mL) in transgenic *Ds-bar* and GP-*bar* lines, as determined by enzyme-linked immunosorbent assay (ELISA)

Line	Total protein of tested extract (mg/mL)			
	0.0625	0.125	1.0	2.0
GP- <i>bar</i> 59*	1367	945	–	–
GP- <i>bar</i> 30†	–	–	123	–
<i>Ds-bar</i> 11‡	1350	887	–	–
<i>Ds-bar</i> 19‡	1641	948	–	–
<i>Ds-bar</i> 21*	1061	789	–	–
<i>Ds-bar</i> 1†	–	–	14	25
<i>Ds-bar</i> 6†	–	–	44	41

\*F<sub>1</sub> hybrids with Oregon Wolfe Barley Dominant (OWBD) had intermediate viability.

†F<sub>1</sub> hybrids with OWBD were viable.

‡F<sub>1</sub> hybrids with OWBD were viable.

lines showed high levels of PAT (average absorbance value of 2.58 at 0.44 mg/mL total plant protein). If the inviability observed in *Ds-bar* lines 11, 18, 19 and 21 had been caused by a common genetic factor that was absent in *Ds-bar* line 24, all *Ds-bar* line 24 derivatives would have been expected to be viable regardless of their levels of *bar* expression.

These data indicated that the lines that formed viable hybrids with OWBD had less than 100 ng PAT/mg total plant protein vs. approximately 1000 ng PAT/mg total plant protein for lines that formed inviable hybrids (Table 1). These values should be considered as semi-quantitative. The detection of PAT was influenced by the amount of total plant protein, and the wide range of absorbance values between high- and low-expressing lines prevented the performance of meaningful assays at a single protein concentration.

#### Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) assays

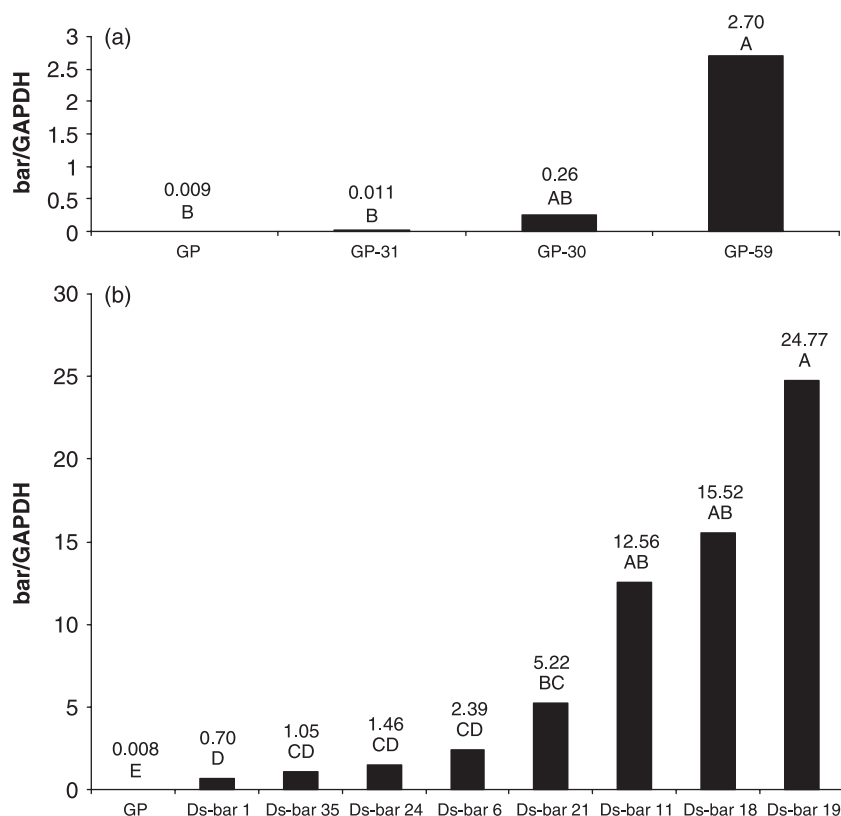
The quantification of *bar* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcription was based on comparisons of sample C<sub>t</sub> values with the C<sub>t</sub> values of the respective standard curves. The quantification of *bar* transcription was normalized against that of GAPDH. Thus, in the context of the qRT-PCR assays, *bar* expression data are presented as the ratio of *bar*/GAPDH transcription. For both *bar* and GAPDH, R<sup>2</sup> values for the standard curves were 0.99 or higher. The slopes for the *bar* standard curves averaged –3.40 (97.2% efficiency; range of –3.23 to –3.59, 90.0%–103.8% efficiency). The slopes for the GAPDH standard curves averaged –3.21 (104.9% efficiency; range of

–3.32 to –3.07; 100.0%–111.6% efficiency). All reported data were derived from assays in which the no-template and no-reverse transcriptase controls either had no recorded C<sub>t</sub> values, or in which the C<sub>t</sub> values for these controls were clearly higher than those recorded for the tested samples with the lowest C<sub>t</sub> values. No formal experiments were conducted to examine the potential existence of inhibitors of the PCRs. However, examination of the variability of the concentrations between undiluted RNA preparations relative to the variability between C<sub>t</sub> values showed no relationship, indicating a lack of significant inhibition.

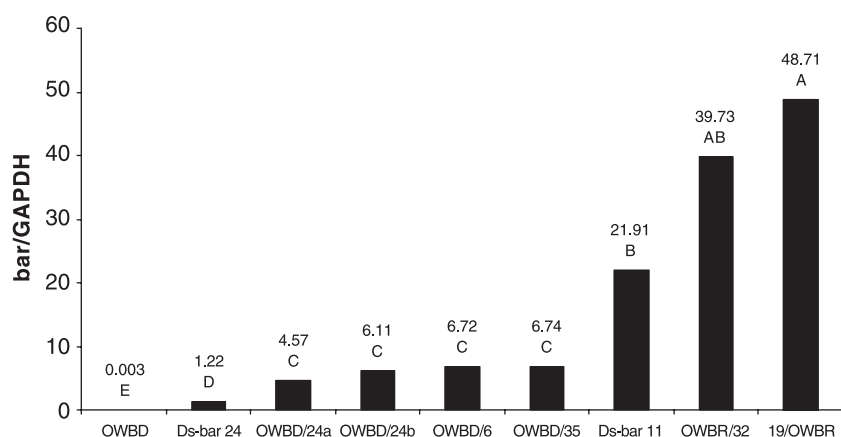
Substantial differences in *bar* transcription were noted between the *Ds-bar* (three experiments) and GP-*bar* (two experiments) lines. Analysis of variance, using a model in which the effect of line was fixed and the effects of experiment and sampling date were random, showed that the effects of both line and line by experiment interaction were significant. Tukey's analyses were conducted on line means, using the line by experiment interaction as the error term. The results of these analyses are shown in Figure 1. These data are consistent with the phenotypic and immunological data presented above. Lines that produced inviable hybrids with OWBD (*Ds-bar* lines 11, 18 and 19) had the highest levels of transcription. Lines that produced hybrids with intermediate viability (*Ds-bar* 21 and GP-*bar* 59) had higher levels of *bar* transcription than lines that produced viable hybrids (*Ds-bar* lines 1, 6, 24 and 35; GP-*bar* lines 30 and 31).

Several different experiments were conducted to investigate *bar* transcription in hybrid progeny derived from various crosses with *Ds-bar* lines. Comparisons of transcript levels between lines were made using analysis of variance as described above. Backcross 1-derived hybrid progeny of OWBD and *Ds-bar* lines 6, 24 (lines a and b were derived from two different selections) and 35 showed relatively low levels of *bar* transcription, similar to that of *Ds-bar* line 24 and less than that of *Ds-bar* line 11 (Figure 2).

It was not possible to measure the *bar* transcription of inviable hybrids, as the tissues were not recovered from these plants before they became necrotic. However, high levels of *bar* transcription in hybrid progeny of OWBD and *Ds-bar* lines could be inferred by establishing that parental levels of *bar* transcription were heritable. The heritability of *bar* expression has been demonstrated by phenotypic studies of hybrid progeny of GP-*bar* lines 30, 31 and 59 when crossed with a genetically diverse set of two-rowed and six-rowed barley genotypes (Bregitzer and Tonks, 2003). The current study also demonstrated that both high and low parental levels of *bar* transcription were heritable on a relative basis. Comparisons of transcript levels between lines, based on analysis of



**Figure 1** Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analyses of *bar* expression, relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, in seedling leaf tissues of GP-*bar* (a) and *Ds-bar* (b) transgenic lines, and non-transgenic Golden Promise. Numbers over each bar represent the mean values derived from two experiments for GP-*bar* lines and three experiments for *Ds-bar* lines. Mean values followed by the same letter are not significantly different ( $P = 0.05$ ), as determined by Tukey's analysis.



**Figure 2** Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analyses of *bar* expression, relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, in 27-day-old leaf tissues of  $BC_1F_2$ -derived  $F_3$  hybrids of various *Ds-bar* lines and Oregon Wolfe Barley Dominant (OWBD), and  $F_2$ -derived  $F_3$  hybrids of *Ds-bar* lines and Oregon Wolfe Barley Recessive (OWBR). Numbers over each bar represent the mean values derived from two experiments. Mean values followed by the same letter are not significantly different ( $P = 0.05$ ), as determined by Tukey's analysis.

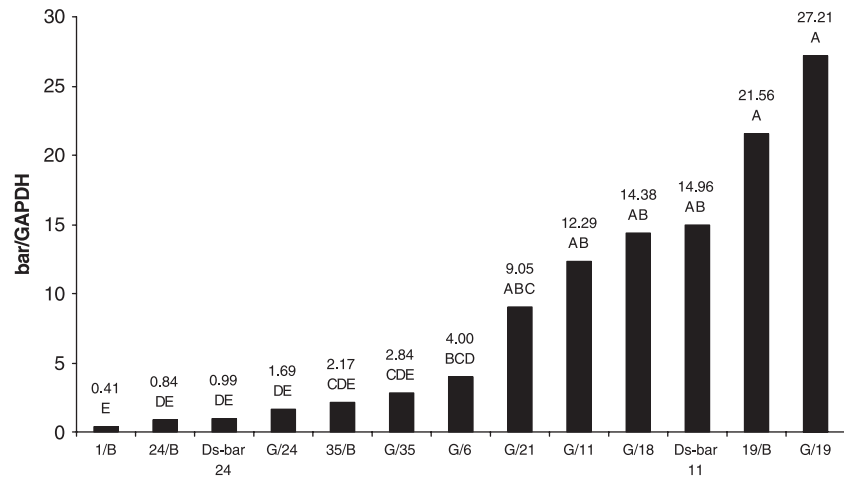
variance as described above, showed that the observed relationships between the parental *Ds-bar* lines for *bar* transcription also existed for hybrid progenies of crosses with OWBR, Garnet and/or Baronesse (Figures 2 and 3; note that Figure 3 includes data for the progeny of a cross with *Ds-bar* line 32; this line was not analysed for *bar* transcription, but OWBD/*Ds-bar* line 32 hybrids are inviable). These data were consistent with the ELISA assessments of PAT concentrations. Thus, it is reasonable to infer high levels of *bar* expression in

the hybrid progeny derived from crosses of OWBD with high-*bar*-expressing *Ds-bar* and GP-*bar* lines.

### Genetic characterization and mapping of inviability

The high-*bar*-expressing *Ds-bar* line 19 was crossed with 88 of the 94 doubled haploid lines that comprise the OWB mapping populations (Costa *et al.*, 2001). Hybrid seed was recovered from all crosses, but seedlings from two crosses

**Figure 3** Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analyses of *bar* expression, relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, in 17-day-old leaf tissues of *Ds-bar* lines and in  $F_3$ -derived  $F_4$  progeny populations, homozygous for their respective *Ds-bar* inserts, derived from crosses of various *Ds-bar* lines with Garnet (G) and/or Baroness (B). Each  $F_3$ -derived  $F_4$  progeny population is represented by two lines that were derived from different  $F_2$  plants. Numbers over each bar represent mean values derived from two experiments. Mean values followed by the same letter are not significantly different ( $P = 0.05$ ), as determined by Tukey's analysis.

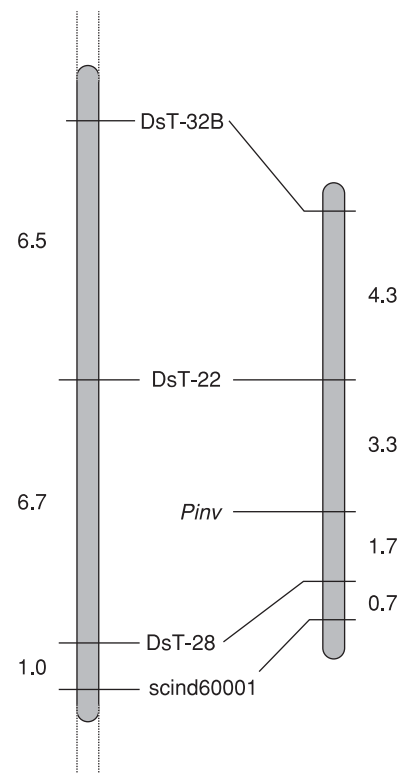


did not germinate. ELISAs conducted on seedling tissues indicated high PAT expression (including for some inviable lines from which tissues were obtained prior to death). Similarly high absorbance values to those seen for *Ds-bar* line 19 were observed (data not shown). Visual scoring for hybrid viability was conducted 2–3 weeks after germination, and the results were consistent with the presence of a single locus: 44 progeny lines were viable and 42 were inviable.

We designated this locus as *Pinv* (PAT inviability) and placed it on the 232-marker OWB map (Cooper *et al.*, 2004) via multipoint linkage analysis using MAPMAKER 3.0 (Lander *et al.*, 1987). This analysis showed that *Pinv* mapped to chromosome 6HL, and was linked to the flanking markers DsT-22 (at an LOD [log of the odds] of 11.6) and DsT-28 (at an LOD of 15.6) (Figure 4).

## Discussion

These data clearly show that the expression of *bar*, at least above certain levels, is not compatible with the normal physiological processes of OWBD. Therefore, for OWBD, *bar* is not suited for use as a neutral selectable marker nor as a means to confer herbicide resistance. However, other genotypes showed no such sensitivity. In the current study, the negative effects of *bar* expression were not detected in the Golden Promise *Ds-bar* line, nor in hybrids of *Ds-bar* lines with Baroness, Garnet and OWBR. No previous reports associating *bar* expression with any type of physiological disturbances in any crop are known to the authors. Furthermore, extensive and detailed evaluations have been conducted, including replicated field tests, of *bar*-expressing transgenic barley lines in numerous cultivars and breeding lines that represent a diversity of genetic backgrounds (Bregitzer *et al.*, 1998; Bregitzer and Tonks, 2003; P. Bregitzer and L.S. Dahleen,



**Figure 4** Linkage maps showing the position of phosphinothricin acetyltransferase inviability (*Pinv*). The linkage map on the left is derived from the Oregon Wolfe Barley (OWB) map reported in Cooper *et al.* (2004); the map on the right is from this study. The numbers indicate distances between markers in centimorgan (cM) (Haldane).

unpubl. data), and of wheat lines in the background of the cultivar Bobwhite (Bregitzer *et al.*, 2006). None of these studies has detected, nor did the current study detect, any problems associated with *bar* expression, except in OWBD. Furthermore, no yield differences could be detected between high- and low-*bar*-expressing lines (*Ds-bar* lines as well as backcross



derivatives in the Golden Promise and Garnet backgrounds) in 2 years of field testing in replicated small-plot trials (P. Bregitzer, unpubl. data).

These data offer little in the way of explanation of the mechanism responsible for the observed inviability, but it is reasonable to speculate that PAT, in some way, acetylates glutamate – which is a structural analogue of phosphinothricin (glufosinate) – in OWBD. The observation of slow growth and progressive chlorosis is consistent with impaired nitrogen metabolism via reduced assimilation of ammonium, such as has been shown in barley leaves as glutamine synthetase becomes down-regulated as part of normal senescence (Weidemuth *et al.*, 2005). Detailed genetic investigations of *Pinv* – the locus associated with PAT-induced inviability in this study – in conjunction with studies of nitrogen metabolism in OWBD are necessary to investigate this possibility.

It is important to note that OWBD is genetically dissimilar to other cultivated barley genotypes. Rostoks *et al.* (2005) presented a diversity assessment that indicated that OWBD was as unrelated to two groups of barley (*Hordeum vulgare* spp. *vulgare*) cultivars (European and North American heritage) as it was to an accession of wild barley (*H. vulgare* ssp. *spontaneum*). This is not surprising, given that many key OWBD traits are derived from genetic stocks containing allelic variation that is agronomically undesirable. Such variation has been systematically selected against during the development of cultivated barley. The brittle locus (*Btr1*) and the dwarfing locus (*Zeo*) mutations present in OWBD are examples of allelic variation not found in any modern barley cultivar. The *Btr1* locus is also unique in that it must have been derived from crosses with wild barley that are in the OWBD pedigree. It is possible that the region of chromosome 6HL associated with PAT-induced inviability contains variability that is atypical of most or perhaps all cultivated barley genotypes.

The wider implications of the observations reported in this study are unclear. Certainly, they demonstrate the presence of allelic variability that has not been detected previously within barley or other cereal species. Should this variability involve an important physiological pathway, such as nitrogen metabolism, investigations of OWBD may facilitate further discoveries that could lead to novel and useful applications. Perhaps the potential problem of PAT-induced inviability should be considered when contemplating the transformation of wild *Hordeum* species, or when searching for solutions to failed efforts to recover transgenic plants from cultivated barley genotypes not known to be tolerant of PAT. However, given the apparent restriction to a single, unrepresentative genotype, this phenomenon may have little practical consequence, except to restrict the utility of *bar* in OWBD,

and, hopefully, to remind us of the complex and sometimes surprising aspects of higher plant genomes.

## Experimental procedures

### Plant materials

Four groups of plant material were used for these experiments: (i) transgenic lines (*Ds-bar* lines) containing transposed *Ds* inserts which comprised a ubiquitin-driven *bar* expression cassette flanked by maize *Ds* terminal sequences (Koprek *et al.*, 2000; Cooper *et al.*, 2004); (ii) transgenic lines (*GP-bar* lines) derived from unrelated transformation events which contained ubiquitin-driven *bar* expression cassettes but lacked *Ds* sequences; (iii) non-transgenic lines, including the OWBD and OWBR multiple marker genetic stocks and a 94-line doubled haploid mapping population derived from a cross of these parents (Costa *et al.*, 2001), and the cultivars Golden Promise, Garnet and Baroness; and (iv) hybrid progeny lines derived from crosses of various *Ds-bar* or *GP-bar* lines with OWBD, OWBR, Garnet and/or Baroness. Both the *Ds-bar* and *GP-bar* lines were produced in the Golden Promise background, and each line was homozygous for its respective *bar* locus.

The *Ds-bar* lines were secondary transposition events (Singh *et al.*, 2006) derived from crosses of a common transgenic *Ds-bar* parent with transgenic lines expressing maize *Ac transposase*, and each line had a *Ds-bar* locus in a unique position that had been delivered via *Ac transposase*-mediated transposition. As tested, they were *Ac transposase*-negative segregants that had been advanced to the F<sub>4</sub> or F<sub>5</sub> generation using single-seed descent. All exhibited resistance to application of 0.05% glufosinate-ammonium. Eight lines were selected as the primary focus of the present study based on the viability of their progeny when crossed with OWBD. OWBD/*Ds-bar* line 1, 6, 24 and 35 hybrids grew well and survived to produce mature and viable seed. OWBD/*Ds-bar* line 11, 18 and 19 progeny grew poorly and died in the seedling stage. The OWBD/*Ds-bar* line 21 hybrid survived to the tillering stage, but died prior to flowering. Eight additional *Ds-bar* lines, each a unique tertiary transposition event derived from *Ds-bar* line 24 (Singh *et al.*, 2006), were also studied. Hybrid progenies resulting from crosses of these *Ds-bar* line 24 derivatives were not viable.

The *GP-bar* lines were derived directly from T<sub>0</sub> plants developed and described by Wan and Lemaux (1994), advanced to the T<sub>5</sub> or T<sub>6</sub> generation via single-seed descent. All had been extensively characterized in earlier generations for *bar* expression via phenotypic assays of resistance to glufosinate-ammonium (Bregitzer and Tonks, 2003). GP717B-59-11 ('GP-bar 59') exhibits a high level of resistance to glufosinate-ammonium damage, GP724B-4-9 #31 ('GP-bar 31') exhibits a very low level of resistance and GP724B-4-9 #30 ('GP-bar 30') exhibits an intermediate level of resistance. The analysis of recombinant populations in several different genetic backgrounds showed that the relative levels of resistance were heritable (Bregitzer and Tonks, 2003). Northern analyses have shown detectable levels of *bar* messenger RNA (mRNA) in *GP-bar* 30, but not in *GP-bar* 31 (Meng *et al.*, 2003); no such characterizations have been conducted for *GP-bar* 59. Images of leaves from each of these lines after treatment with 0.05% glufosinate-ammonium, which illustrate their resistance phenotypes, are published in Bregitzer and Tonks (2003).

All plants were grown in a glasshouse using a sand–peat–vermiculite (in approximately equal ratios) mix supplemented with a slow-release fertilizer (Osmocote 19-5-2 plus essential micronutrients). Irrigation was supplied by overhead sprinklers. These experiments were conducted over several years in all seasons except for mid-summer, when the glasshouse cooling systems were inadequate for the needs of barley plants. The temperature ranged from approximately 10 °C at night to 20–30 °C during the day. During the winter months, supplemental light was provided with metal halide bulbs to extend the day length to at least 14 h.

### Immunological analysis

PAT protein was extracted in phosphate-buffered saline (PBS)–Tween from fresh, single leaves of 4–16-week-old seedlings. PAT was detected using a sandwich ELISA kit as directed by the manufacturer (SDI, Dow AgroSciences Quantitative PAT/BAR Microtiter Plate ELISA Test Kit, p/n 7140200; Dow AgroSciences, Newark, DE). Purified PAT protein was supplied by SDI and used to establish the standard curves. Standard curves (0.25–4 ng/mL) were prepared in PBS or in non-transgenic GP total protein extracts (0.0625, 0.125, 1.0 or 2.0 mg/mL). The total protein extracts of the assayed lines were diluted to between 0.0625 and 0.5 mg/mL for high-expressing lines and between 0.25 and 2.5 mg/mL for low-expressing lines to keep the optical density readings within instrument limits. Plates were read on a DYNEX (Chantilly, VA, USA) MRXII plate reader.

### qRT-PCR

For comparisons between *Ds-bar* and GP-*bar* lines, leaf tissue samples were collected from single plants, grown in the spring of 2005, 32 and 53 days after planting. A third set of samples, grown in the autumn of 2005, were collected from multiple (9–11) plants, 29 days after planting. For comparisons between  $F_3$ -derived  $F_4$  hybrids from crosses of *Ds-bar* with Garnet or Baronesse, leaf tissue samples were collected from multiple (9–11) plants, grown in the spring of 2005, 17 days after planting. For comparisons of  $BC_1F_2$ -derived  $F_3$  and  $F_2$ -derived  $F_3$  hybrids from crosses of *Ds-bar* lines with OWBD or OWBR, leaf tissue samples were collected from multiple (9–11) plants, grown in the autumn of 2005, 27 days after planting. Leaf tissues were frozen immediately after collection with liquid nitrogen, and stored at –80 °C prior to RNA extraction. RNA was extracted using the Qiagen (Valencia, CA, USA) RNeasy Purification Kit with RNase-free DNase I on-column digestion, as specified by the manufacturer. Quantification and purity assessments of RNA concentrations were determined using spectrophotometric measurements of absorbance at 260 and 280 nm. Only samples with  $A_{260}/A_{280}$  ratios of 1.9–2.1 were used for these experiments. RNA samples were diluted to 150–200 ng/μL.

Complementary DNA (cDNA) was synthesized from 1 μg of total RNA in 20-μL reactions using the ThermoScript III-cDNA First Strand Synthesis Kit, including RNase H (Invitrogen, Albany, CA, USA), employing oligo dT primers, according to the protocols provided with the kit. Initial priming was conducted at 68 °C for 5 min, followed by incubation at 55 °C for 55 min, deactivation by heating to 85 °C for 5 min, cooling on ice for 5 min, and treatment with RNase H. Each cDNA preparation was then diluted by adding 20 μL

of Tris-EDTA (TE); 2 μL of each of these dilutions was used for each qRT-PCR. A no-reverse transcript control reaction was prepared from the *bar*-expressing *Ds-bar* line 19 as above, except for the exclusion of reverse transcriptase from the reaction mix.

Standard curves for *bar* and GAPDH were constructed using templates derived from linearized plasmid containing a *bar* expression cassette (*pSPDsbar*; Koprek *et al.*, 2000) or from an amplified barley GAPDH cDNA fragment. The GAPDH fragment was 761 bp in length and was produced using the forward primer TGCTGATTACGTTGT-GGAATCC and the reverse primer AGGCGCCACAAGGAAAGC. These primers were designed based on the barley GAPDH sequences deposited with GENBANK (Accession #X60343, Hv GAPDH coding). Dilutions for the standard curves ranged from approximately  $4.4 \times 10^{-5}$  to  $2.7 \times 10^{-2}$  pg for *bar* and from approximately  $3.2 \times 10^{-5}$  to  $2.0 \times 10^{-2}$  pg for GAPDH (based on the masses of the target sequences).

qRT-PCR analyses were carried out using primers (Operon Biotechnologies, Inc., Huntsville, AL, USA) near the 5' end of *bar* and GAPDH. For *bar*, the forward primer was TCTGCACCATCGTCAACCA (starting 63 bases downstream of the start codon) and the reverse primer was GATAGCGCTCCCGCAGAC; the amplified product was 111 bp in length. For GAPDH, the forward primer was TGCTGAT-TACGTTGTGGAATCC (starting 279 bases downstream of the start codon) and the reverse primer was TGGCACCACCTTCAAGTG; the amplified product was 77 bp in length. Labelled probes were synthesized (Sigma Genosys, St. Louis, MO, USA) and labelled with FAM-5' (6-carboxy-fluorescein) reporter and TAMRA-3' (tetramethyl-6-carboxyrhodamine) quencher. The probe sequences were CCACTCCT-GCGGTTCTGCGG (*bar*) and CACTGACAAGGACAAGGCCGCTGC (GAPDH). The primers and probe for GAPDH were targeted to regions of high GC content to achieve similar  $T_m$  values to the primers and probe used for *bar*. Primer and probe sets were designed using the ABI Prism 7000 SDS Primer Express software (Applied Biosystems, Foster City, CA, USA).

PCR assays were performed using Sigma Jump-Start Taq Ready Mix (Sigma-Aldrich, St. Louis, MO, USA), optimized for  $Mg^{2+}$  concentration, employing the QRT Stratagene MX 3005P Real Time PCR system. The resulting data were analysed using the software provided (Stratagene, La Jolla, CA, USA). The reactions were initiated by incubating at 95 °C for 4 min, followed by 45 cycles of 95 °C (45 s)/64 °C (55 s). Each assay consisted of triplicate reactions for each tested line, and duplicate reactions for the *bar* and GAPDH standard curves. Both *bar* and GAPDH amplifications were run simultaneously on the same plate. No-reverse transcriptase cDNA preparations and no-template controls were included for each assay.

### Mapping analysis

*Ds-bar* line 19 was crossed with each of the DH lines of the OWB mapping population (Costa *et al.*, 2001). Seven seeds of each  $F_1$  progeny line were planted in the glasshouse and scored for viability. Seedlings and plants that developed vigorously and appeared to be normal, healthy barley plants were scored as 'viable'; seedlings that failed to develop normally or that died soon after germination or emergence were scored as 'inviable'. PAT activity was assessed in all  $F_1$  progeny lines via ELISAs (as described above).

The locus associated with PAT-induced inviability (*Pinv*) was mapped using MAPMAKER/EXP 3.0 (Lander *et al.*, 1987) analysis of the most recent version of the OWB map containing 232 markers (Cooper *et al.*, 2004; November 9, 2005 update of the data set

available at <http://barleyworld.org/oregonwolfebarleys/maps.php>). The initial placement of *Pinv* was based on three-point linkage analysis, and the final resolution of the map position was based on multipoint linkage analysis, using the Haldane centimorgan function.

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